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This grant proposal is to synthesize potential KGF antagonists which will then be evaluated for efficacy in *in vitro* assay systems. The results generated for the proposed study will be useful for designing new therapeutic agent for breast cancer. KGF antagonist candidate peptides have been synthesized and evaluated its ability. There is, however lack of sensitivity issue underlies on the functional assay procedure. We then developed several KGF-receptor overexpressed cell lines for further use. WE are on the process to evaluate the cell lines.

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FOREWORD

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Introduction:

This grant proposal is to synthesize potential KGF antagonists which will then be evaluated for efficacy in *in vitro* assay systems. The results generated for the proposed study will be useful for designing new therapeutic agent for breast cancer.

Body:

Statement of Work

Development of KGF antagonist as a breast cancer therapeutic agent

Task 1: Synthesis of nine hexacosapeptides as KGF antagonists (Months 1-2)

As reported in the year-1 progress report, KGF antagonist candidate peptides have been synthesized. Stably-transfected MCF-7 and MDA-MB-231 cells with KGF were also established. The stably transfected MCF-7 cells with KGF (MCF-7-KGF) were used for a ^{3}H-thymidine incorporation assay and aromatase activity assay. However, significant inductions of ether DNA-synthesis or aromatase activity were not observed by KGF treatment. The stably transfected MDA-MB-231 cells with KGF (MDA-MB-231-KGF) were also examined, however, significant positive induction of either DNA-synthesis or aromatase activity were not observed by KGF treatment. A receptor-binding assay was also performed using MCF-7-KGF, however, neither a competitive binding assay nor cross-linking assay drowned a reportable positive result. The P.I., then concluded that a reason of these series of failures might be a lack of sensitivity, in other word, low KGF-receptor (KGFR) expression in those cells. Therefore, we decided to establish a KGFR overexpressed breast cancer cell line. A mouse KGFR cDNA cloned in a mammalian expression vector was obtained from Dr. Pui-Kai Li, The Ohio State University, and the plasmid was transfected into the NIH-3T3 mouse fibroblast cells, MCF-7, MDA-MB-231 and T47D breast cancer cells. Currently, transfected cells are in a process of G-418 antibiotics selection. To select a higher KGFR expressed stably transfected cells, KGFR extra cellular region specific antibody was raised in a rabbit. This KGFR specific antibody will be *Cy3*-labeled and be applied a flow cytometry analyses. The cell surface levels of KGFR protein in the transfected cells will be evaluated by using this method. Most effectively expressed cell line will be used for further experiments. The KGFR specific peptide used for this purpose was as follows: HSGINSSNAE. This amino acid sequence is a part of the KGFR specific region, 38 amino acids as follows: (HSGINSSNAEVLALFNVTEADAGEYICKVSNYIGQANQ). This sequence was analyzed by computer software with the following factor as parameters and narrowed to a suitable 10 amino acid for the antisera production: Hydrophyilicity: Kyte-Doolittle, Antigenic index: Jameson-Wolf, Surface probability: Emini, Flexible regions: Karplus-Schulz, Alpha-Amphyipathic region: Eisenberg, Beta-Amphyipathic region: Eisenberg. Alpha, beta, turn, or coil resions: Garnier-Robson, and Chou-Fasman. KGFR over expressed protein: The extra cellular domain of KGFR cDNA was RT-PCR-cloned by using the following pair of primers: 5'-ACCATGGTCAGCTGGGGTCGTTTCATCTGCCT-3' and 5'-CTCCAGGTAGTCTGGGGAAGCTGTAATCTCC-3'. The amplicons of KGFR extra cellular domain CDNA was cloned into a mammalian gene express vector pEF6/V5-His-TOPO. A clone was DNA sequenced and verified the orientation. This clone, pEF6/V5-His-KGFR, was used for the establishment of stably transfected cells with KGFR-overexpression plasmid (pEF6/V5-His-KGFR). A human breast cancer cell line T47D

was transfected with the pEF6/V5-His-KGFR plasmid and then the stably transfected cells were selected by using the Blasticidin antibiotics. The overexpressed cells were cultured and the condition medium was collected twice a week to pool. The pooled medium was used for the overexpressed KGFR-extracellular membrane portion purification. Since the overexpressed protein has a stretched His (6xHis) in the –COOH terminal of its protein, it was purified by using a Nickel-Chelationg resin. The purified protein molecular size was then analyzed by using SDS-PAGE. The molecular size (MW: about 68,000) was, however, larger that the expected (MW: 45,000). It is known that most of FGFRs are highly glycosylated. We analyzed the protein whether the protein is glycosylated by using the glycosylation protein detection kit (BioRad). And as shown in Fig 1, the overexpressed protein was glycosylated. Subsequently glycoprotein profiles analysis was performed by using the Enzyme Deglycosylation kit (BioRad). As shown in Fig 2, there are two major bands are seen at sizes of 68,000 and 45,000. These bands are originally expressed protein (MW 62,000) and sugar free (enzymaticaly hydrolysis of oligosaccharids, MW 36,000), respectably. However, further carbohydrate analysis has not been performed. We then, used this overexpressed protein for receptor binding assay studies. We found that the ^{125}I-labeled KGF binds to this protein. However, the size of ligand-ligan complex is not as expected. We are currently in a process to analyze this data whether the complex is ^{125}I-labeled KGF –KGFR complex or not.

Task 2: KGF peptide Three-D structural analysis (Months 1-3)

This task is in progress. Dr. Robert W. Brueggemeier, a coinvestigator and his colleagues has presented their progress at the 93rd Annual meeting of American association for Cancer Research. (Virtual screening using a KGFR homology model, John C. Hackett, Pui-Kai Li, Robert W. Brueggemeier). Please see an attached copy.

Task 3: Syntheses of a series of small peptides based on 3-D structural analysis results (Months 4-5)

The first molecule that has identified as a responsive element to bind to KGFR, (ArgThrValAlaVal), was synthesized and its biological activity is being tested. After this series of evaluation is completed, following task will be persuaded.

Task 4: Syntheses of a series of modified small peptides (Months 18-20)

This task has not been started, however as soon as task 3 is completed this will be started.

Key Research Accomplishments

Extracellular portion of KGFR protein was overexpressed in a human breast cancer cells and purified for further studies.

Carbohydrate analyses of purified protein were performed.

Stably transfected cells with KGFR were established and be applied for the further experiments.

Reportable outcomes

Chang HL, Sugimoto Y, Liu SL, Jiang JH, Kulp SK, Brueggemeier RW, Lin YC, Development of an in vitro model for the screening of biologically active keratinocyte growth factor (KGF/FGF-7) receptor antagonists., BIOLOGY OF REPRODUCTION 64: 166-166, Suppl. 1 2001

Sugimoto Y, Kashida Y, Chang HL, Brueggemeier RW, Lin YC, Characterization of a metastasis supplessor genes CC3/TC3 in breast cancer cells, 93rd annual meeting for the American Association for Cancer Research.

John C. Hackett, Pui-Kai Li, Robert W. Brueggemeier, Virtual screening using a KGFR homology model, 93rd annual meeting for the American Association for Cancer Research.

Conclusions

Extracellular portion of KGFR protein was overexpressed in a human breast cancer cells and purified for further studies.

Referrences

Chang HL, Sugimoto Y, Liu SL, Jiang JH, Kulp SK, Brueggemeier RW, Lin YC, Development of an in vitro model for the screening of biologically active keratinocyte growth factor (KGF/FGF-7) receptor antagonists., BIOLOGY OF REPRODUCTION 64: 166-166, Suppl. 1 2001

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John C. Hackett, Pui-Kai Li, Robert W. Brueggemeier, Virtual screening using a KGFR homology model, 93rd annual meeting for the American Association for Cancer Research.

Appendices

Figure 1. Detection of glycosylated protein

The overexpressed extracellular portion of KGFR protein (1μg) from 14 individual clones were loaded to PVDF membrane and glycosylated protein detection was performed accordingly to the kit mamual instruction. Gray dots are positive to glycosylated protein. All protein loaded to the membrane shown to be positive.

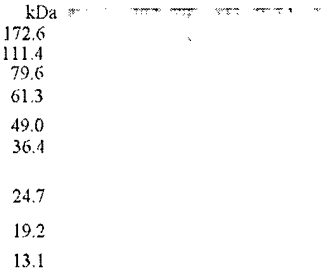

Figure 2. Enzymatic deglycosylation of overexpressed extracellular portion of KGFR protein

A 2μg of overexpressed extracellular portion of KGFR protein from four individual clones were subjected for enzymatic deglycosylation analysis. Two bands are shown on the SDS-PAGE gel. The upper band around 61kDa is the original protein size and lower band around 36kDa is the deglycosylated protein.

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Virtual screening using a KGFR homology model

John C. Hackett, Pui-Kai Li, Robert W. Brueggemeier, The Ohio State University, Columbus, OH.

KGF (Keratinocyte Growth Factor) is strongly mitogenic in vitro for a broad range of epithelial cells from different organs, including the mammary gland. KGF is the first member of the fibroblast growth factor family to be found at similar levels in breast cancers and nonmalignant tissues. It is expressed in stromal fibroblasts and is capable of stimulating cell proliferation of breast cancer cells in which the expression of its receptor FGFR2-IIIb (KGFR) seems to be retained. We have developed a homology model of the human KGFR using computational techniques. The FGFR1 tyrosine kinase domain has been solved as the apoprotein and complexed with inhibitors, and shares 86% identity with the analogous domain in KGFR. In silico site-directed mutagenesis was used to generate a crude mode of the KGFR receptor tyrosine kinase domain. This was immersed in a box of TIP3P water and subjected to a global molecular dynamics simulation using the AMBER 4.1 force field until the atoms reached a stable trajectory. Time averaged structures were generated by averaging from the point a stable trajectory was obtained until the radius of gyration began to decrease. Average structures were subjected to minimization to a gradient of 0.5 kcal/angstrom, followed by 100 steps of steepest descent minimization. Our laboratory has developed a method to construct benzopyranone libraries using a solution phase and resin capture strategy. We also have access a library of 3-phenyl quinolinones. Analogs of both of these compounds have been shown to have activity against a number of tyrosine kinases. Presently, we are using FlexX within the Sybyl 6.7 environment to perform virtual screening of the small molecule libraries to identify putative KGFR tyrosine kinase inhibitors. The overall goal is to develop a selective KGFR tyrosine kinase inhibitor to study how KGF controls growth in breast cancer, and identify a novel therapeutic agent.